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Changes in the CD44 variant (CD44v) isoforms on the cell surface have been correlated with tumor metastasis. In this study we have examined the expression of CD44 variant isoforms in human breast carcinoma samples by a variety of techniques including immunohistochemistry, reverse transcriptase-polymerase chain reaction (RT-PCR) and nucleotide sequencing. Using RT-PCR, we have determined that normal human breast tissue contains primarily the CD44 epithelial (CD44E) form and very little CD44 standard (CD44s) form. However, metastatic breast carcinomas appear to overexpress both the CD44E and CD44s forms and also display multiple new species of CD44 variant isoforms. Histocytochemical staining using anti-CD44 antibody (recognizing a common determinant of the CD44 class of glycoproteins) confirms that the CD44 molecules are overexpressed and preferentially located in infiltrating ductal carcinomas (IDC) and/or infiltrating lobular carcinomas (ILC) obtained from metastatic breast cancer tissues. Nucleotide sequencing analyses indicate that at least four, new CD44 variant isoforms (i.e. displaying unique splicing via the insertion or the deletion of exons 7, 10, 11 and 14) may be closely associated with human metastatic breast cancers. These newly described CD44 variant isoforms may be useful for monitoring the progression of human breast cancer metastasis.

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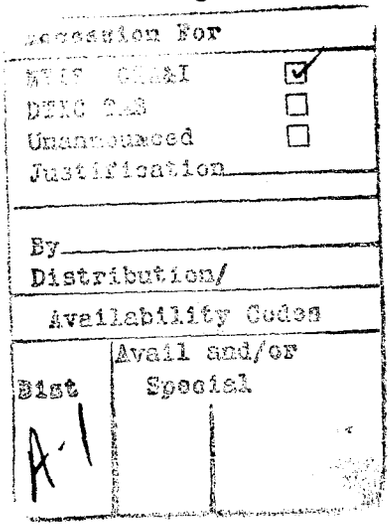

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INTRODUCTION

The transmembrane glycoprotein, CD44 has been shown to bind extracellular matrix components such as hyaluronic acid (HA) at its N-terminal domain (Underhill, 1992; Bourguignon, et al., 1993) and to contain an ankyrin binding site within its 70 amino acid long C-terminal domain (Kalomiris and Bourguignon, 1988; Lokeshwar and Bourguignon, 1991; Bourguignon, et al., 1993; Lokeshwar, et al., 1993). It has been detected in a variety of cell types including, T and B lymphocytes, macrophages monocytes, granulocytes, erythrocytes, fibroblasts, epithelial cells, endothelial cells, smooth muscle cells, retina tissues and a subset of glial cells in the central nervous system (Haynes, et al., 1989; Picker, et al., 1989; Bourguignon, et al., 1992; Salmi, et al., 1993; Chaitin, et al., 1994). CD44 has also been shown to be a novel GTP-binding protein (Lokeshwar and Bourguignon, 1992) which responds to protein kinase C-mediated phosphorylation (Kalomiris and Bourguignon, 1989) as well as acyl-transferase-mediated acylation (Bourguignon, et al., 1991). Due to its widespread occurrence and its role in signal transduction, CD44 has been suggested to play an important role in the regulation of cell activation and cell-cell/cell-extracellular matrix interactions.

Recent studies indicate that the expression of CD44 on the cell surface changes profoundly during tumor metastasis, particularly during the progression of various carcinomas. In fact, CD44 expression has been used as an indicator of metastasis (Arch, et al., 1992; Matsumura and Tarin, 1992; Screaton, et al., 1992; Heider, et al., 1993; Tanabe, et al., 1993; Gunthert, et al., 1991; Rudy, et al., 1993; Tolg, et al., 1993; Penneys and Shapiro, 1994). Sequencing analysis reveals that CD44 exists in different forms (at least 15 isoforms have been identified) that are variants of the common or standard hemopoietic form, CD44s (Arch, et al., 1992; Screaton, et al., 1992; Tolg, et al., 1993). Specifically, the human CD44 gene contains 19 exons, out of which 10 exons can potentially be

alternatively spliced (Arch, et al., 1992; Screaton, et al., 1992; Tolg, et al., 1993). In particular, CD44s contains exons 1-4 (extracellular N-terminal 150 a.a.); exons 5, 15 and 16 (membrane proximal 85 a.a.); exon 17 (transmembrane domain); and a portion of exons 17 and 19 (cytoplasmic tail, 70 a.a.) (Screaton, et al., 1992). The other exons (e.g. 6-14) are also spliced in a variety of different cells. For example, epithelial cells contain exons 12-14 inserted into the CD44s transcripts (Screaton, et al., 1992). This isoform has been designated as CD44E (Screaton, et al., 1992). Other CD44 variants (CD44v) have the same sequences at the two ends of the molecule, but differ in a middle region (with different numbers of additional exons) located on the external side of the cell membrane (Arch, et al., 1992; Screaton, et al., 1992; Tolg, et al., 1993). For example, a certain CD44v is transiently expressed in certain macrophages, along with T- and B-cells, in response to antigen stimulation (Arch, et al., 1992). This finding suggests that CD44v isoforms may play an essential role in the activation of immature lymphocytes into immune cells. Most importantly, Zoller's group recently discovered that during metastasis tumor cells express CD44v structures which mimic developing lymphocytes and allows them to escape immune surveillance.

Breast cancer is currently the most common cancer among women. It is predicted that about 46,000 women will die from breast cancer in 1994-1995. Although the expression of multiple CD44 variant isoforms has been shown to be present in human breast cancer development (Matsumura and Tarin, 1992), it is not clear at the present time, which type(s) of CD44 variant isoform is(are) preferentially expressed in metastatic breast carcinoma tissues. In this study we have used a variety of techniques, including immunohistochemistry, reverse transcriptase-polymerase chain reaction (RT-PCR) and nucleotide sequencing, to analyze the expression of CD44 variant isoforms in human breast cancer tissues. Our data indicate that there is a close relationship between the expression of certain

CD44v's and human breast cancer metastasis. Therefore, the potential usefulness of CD44 variant isoforms as markers for monitoring human breast cancer metastasis is clearly implicated.

BODY (RESULTS):

In this study we have used the RT-PCR technique to analyze the expression of various CD44 isoforms in both normal and metastatic breast tissues. Our results indicate that normal human breast tissues contain primarily two CD44-related PCR products (e.g. one major band and one minor band, Fig. 1 A-D). These two PCR products were subsequently "one-step cloned" into the pCR™ vector from Invitrogen Corp. and sequenced. Our nucleotide sequence data indicate that they represent the CD44 epithelial (CD44E) form and the CD44 standard (CD44s) form, respectively (Fig. 1 A-D). Similar low level of CD44 expression (i.e. CD44E and CD44s forms) was also observed in non-metastatic breast cancer tissues (data not shown). Interestingly, both the CD44E and CD44s forms are overexpressed in metastatic breast carcinomas (Fig. 1 E-K). Furthermore, multiple species of the CD44-related gene products (possibly CD44 variant isoforms) have also been found in metastatic breast tissues (Fig. 1 E-K) and lymph node tissues invaded by breast carcinomas cells (data not shown). These findings are consistent with a previous report of several large size CD44 variant isoforms existing in breast carcinoma cells (Matsumura and Tarin, 1992).

In addition, we have used histocytochemical staining and anti-CD44 antibody (recognizing a common determinant of CD44 class of glycoprotein) (Bourguignon, et al.,1992; Bourguignon, et al., 1993) to determine the localization of CD44 in metastatic breast carcinomas and normal breast tissues. For example, our immunohistocytochemical data indicates that certain CD44 molecules are preferentially located in metastatic breast cancer tissues (Fig. 2A). The immuno-reactive form of CD44 appears to be preferentially located in the plasma membrane region of tumor cells (Fig. 2A).

These immunohisto-cytochemical staining patterns of CD44 in metastatic breast tissues are similar to those reported previously using different anti-CD44 antibodies (Joensuu, et al.,1993; Latham, 1993). Normal breast tissue do not reveal any CD44 staining (Fig. 2B) using an identical concentration of anti-CD44 antibody (5-10 μg/ml). Even though sufficient amounts of RNA transcripts for CD44E and CD44s are present (Fig. 1 A-D) in the normal breast tissues, the inability to detect CD44 molecules (using anti-CD44 antibody at the concentration of 5-10μg/ml) in these tissues suggests that both CD44E and/or CD44s polypeptides must be present in relatively small quantities. Interestingly, under the same immuno-staining conditions, metastatic breast tissues display extensive staining of CD44 suggesting that CD44 molecules may be overexpressed in these breast tumor tissues compared to normal breast tissues. These immunohistochemical data (Fig. 2 A) are consistent with the results obtained from RT/PCR analyses showing the overexpression of CD44E, CD44s and multiple CD44 variant species in metastatic breast tissues (Fig. 1 E-K). Currently, we are in the process of determining whether there is a differential distribution of certain CD44 isoforms (i.e. expression in a single cell type or a variety of cell type) in breast cancer tissues during metastasis.

Furthermore, multiple species of the CD44-related PCR products isolated from metastatic breast cancer tissues have been "one-step cloned" into the pCR™ vector from Invitrogen Corp. and subsequently sequenced. Nucleotide sequence analysis has revealed at least four different CD44 variants displaying unique splice choices of variant exons (Fig. 3 B, C , D & E) between exon 5 and exon 15 of CD44 (Fig. 3 A). The first CD44 variant we have identified [designated as CD44v-I] shows unique splice choices of variable exons in which exon 7 is inserted within CD44E form (Fig. 3 B). The second CD44 variant contains exons 10,11,12,13 and 14 within the membrane proximal variable region [designated as CD44v-II] (Fig. 3 C). The third CD44 variant we have identified

[designated as CD44v-III] shows unique splice choices of variable exons in which exon 12 and 13 are inserted, but exon 14 is spliced out (Fig. 3 D). The fourth CD44 variant analyzed [designated as CD44v-IV] contains a single insertion of exon 14 (Fig. 3 E) within the CD44s form (Fig. 3 G). These four CD44 variants are different from the epithelial form (CD44E) known to contain only exons, 12,13 and 14 (Fig. 3 F). The preferential expression of these CD44 variant isoforms in metastatic breast cancer carcinomas may be very important for cell adhesion, migration, invasiveness and the metastasis of human breast cancers.

CONCLUSION:

CD44 isoforms (e.g. CD44s, CD44E and CD44 variants) represent a class of transmembrane glycoproteins distributed on various types of cells including lymphocytes, macrophages, granulocytes, endothelial cells and epithelial cells (Haynes et al., 1989;Picker, et al., 1989; Stamenkovic et al., 1989; Bourguignon et al., 1986, 1992, 1993; Kalomiris and Bourguignon et al., Lokeshwar and Bourguignon, 1991, 1992). Various CD44 isoforms have recently been linked to tumor dissemination and cancer metastasis (Gunthert et al., 1991; Arch et al., 1992). In this study we have used RT/PCR techniques to illustrate a high level of CD44 isoform expression in metastatic breast carcinomas (Fig. 1 E-K) and a low level of CD44 isoform expression in normal breast tissues (Fig. 1 A-D). Immunohistochemical staining data further support the notion that CD44 is overexpressed in metastatic breast carcinomas (Fig. 2A) and is present at a greatly reduced level in normal breast tissues (Fig. 2B). This data concerning CD44 isoform expression in human breast cancer tissues is consistent with the previous findings reported by Fox et al. (Fox, et al., 1993). However, Fox's group also reported the presence of CD44 in normal breast tissues (e.g. myoepithelial cells, acini and ducts) while our data fails to reveal significant CD44 labeling in the normal breast tissues (Fig. 2B). This

apparent discrepancy in immunohistochemical staining may be due to either the different epitope specificities of anti-CD44 antibodies or the amount of anti-CD44 antibody used in these two studies. Preliminary data in our laboratory indicate that CD44 staining becomes detectable in normal breast tissues if the anti-CD44 antibody concentration is increased to 100μg/ml (10-fold more concentrated than the anti-CD44 antibody used in Fig. 2) during immuno-labeling (data not shown). At this high concentration of anti-CD44, the differences in CD44 expression between normal and cancer tissues become difficult to demonstrate. Therefore, we have decided to use the lower concentrations of anti-CD44 antibody (1-10μg/ml) in order to clearly illustrate the overexpression nature of CD44 in breast cancer tissues (Fig. 2A) compared to that in normal epithelial cells (Fig. 2B).

CD44 has also been shown to be the major hyaluronate receptor (Underhill, 1992) and to function as adhesion molecules (Li, et al. 1993). Cells expressing a high level of CD44 often display enhanced hyaluronic acid binding which increases their migration capability (Horst, et al., 1990; Jalknen, et al., 1991). The high level of CD44 expressed in the breast carcinomas in this study (Figs. 1 E-K and 2A) may promote the binding interaction between tumor cells and extracellular matrix materials (e.g. hyaluronic acid) leading to metastatic cell migration. Furthermore, since carcinomas expressing high levels of CD44 are more malignant than those carcinomas with a low level of CD44 expression (Horst, et al., 1990; Jalknen, et al., 1991), the overexpression of the CD44 phenotype may be useful as a metastatic indicator for a number of carcinomas, including breast cancers.

In addition to overexpression of CD44, a number of tumor cells and tissues have been found to express different CD44 variant (CD44v) isoforms (in addition to CD44E and CD44s) due to an alternative splicing mechanism (Arch, et al., 1992; Matsumura and Tarin, 1992; Screaton, et al., 1992; Heider, et al., 1993; Tanabe, et al., 1993; Gunthert, et al., 1991; Rudy, et al., 1993; Tolg, et al.,

1993). These isoforms, containing extra exon insertions (e.g. exons 6-11) within the CD44 membrane proximal region, have been shown to be associated with tumor metastasis (Gunthert, et al., 1991; Rudy, et al., 1993). At the present time, it is not clear whether there are unique CD44 variant isoforms expressed preferentially in metastatic breast tissues. In this study using RT/PCR and nucleotide sequencing analyses, we have identified at least four, new CD44 variant isoforms (i.e. displaying unique splicing via the insertion or the deletion of exons 7, 10, 11 and 14) in human metastatic breast cancer tissue (Figs. 1 and 3).

Currently, very little is known regarding the structural and functional relationships between CD44 exon insertion/deletion and CD44-mediated biological functions (Bourguignon, et al.,1992; Bourguignon, et al.,1993). The expression of exon 14, which displays extensive O- and N-linked glycosylation sites (Lokeshwar and Bourguignon, 1993), has been shown to be specifically associated with a number of transformed hemopoietic cells (Dougherty, et al., 1991). Therefore, it is possible that modifications of exon 14 in CD44v-I,II,III and IV may affect a number of biological functions, such as adhesion, migration and metastasis. It has been suggested that expression of CD44 variants with the additional exon 10 and exon 11 (CD44v-II) (Fig. 3 C) is responsible for tumor progression and metastasis in several animal tumor model systems (Hofmann, et al.,1991; Salmi, et al.,1993). Therefore, we suggest that expression of CD44v-II may be associated with the general tumor metastatic process.

To our knowledge, the expression of CD44v-I, CD44v-III or CD44v-IV has not been described in any other tumor model systems. Most importantly, these three CD44 variants are present in all the metastatic breast carcinomas tested (n=20) [i.e. representative examples shown in (Fig. 1 E-K). The preferential expression and frequent occurrence of CD44v-I, III and IV in various

metastatic breast cancer tissues suggest that these CD44 variants may be unique for human breast cancers. It is speculated that some of these CD44 isoforms on breast epithelial cells may act as surface modulators to facilitate unwanted growth factor receptor-growth factor interactions and subsequent tumor formation. It is also possible that these CD44 variants may interact with extracellular matrix materials (e.g. hyaluronic acid) such that the breast epithelial cells undergo abnormal mitogenesis and migration. This could be one of the critical steps in breast tumor invasion and metastasis.

Future Work: In the coming years, we plan to investigate the physiological role of these CD44 variants during breast cancer metastasis using a variety of biochemical, molecular biological, cytochemical and cell biological techniques. Specifically, we will prepare a panel of breast cancer-specific CD44 variant cDNA constructs and then transfect these cDNAs into normal breast epithelial cells to induce CD44 variant expression in these cells. The results of these studies will allow us to establish the structure-function relationships between certain CD44 variant expression and breast tumor behavior.

In addition, a larger number of metastatic breast cancer samples will be tested in order to establish a definite relationship between certain CD44 variant isoforms and the progression of human breast cancers. Specifically, we plan to carry out a cytochemical localization of various CD44 isoforms, at both mRNA and protein levels, using state-of-the-art laser confocal microscope in order to determine whether there is a selective distribution (i.e. expression in a single cell type or a variety of cell types) of CD44 variants in breast cancer tissues during metastasis. Once the correlation between CD44 variant formation and tumor behaviors is established, CD44 variant isoforms could potentially be useful markers for establishing the progression of breast cancer development. The specific experiments are described as follows:

(A)Cloning these unique variant exons into standard CD44 (CD44s) cDNA and expressing these constructs in non-metastatic human breast epithelial cells:

The human CD44v-IV cDNA (obtained from Dr. Eugene Butcher, Stanford University and available in our laboratory) has been cloned into pRc/CMV/CD44 for expression in mammalian cells. Preliminary data indicate that exon 14 displays extensive O- and N-linked glycosylation sites which may be important for a number of biological functions (e.g. adhesion, migration and mitogenesis). The procedures for cloning and expression of other human breast tumor CD44 variant proteins (e.g. CD44v-I, CD44v-II and CD44v-III) are described as follows: The RT-PCR primers (i.e. TACATCAGTCACAGACCTGC and ATCCATGAGTGGTATGGGAC) will be used to permit the direct cloning of the amplification products into pRc/CMV/CD44 for expression in mammalian cells. The amplification products will contain unique restriction enzyme sites for Hpa I (476) and Tth I(835). The Hpa I site is also unique in the human CD44s insert of pRc/CMV/CD44s (provided by Dr. Eugene Butcher, Stanford University), while there are two sites for Tth I. Therefore, the amplification products can be inserted directly into pRc/CMV/CD44s which has been partially digested with Tth I and completely digested with Hpa I. This will allow the insertion of the variant sequences directly into the CD44s coding sequence of pRc/CMV/CD44s at precisely the correct location and reading frame. Final constructs containing full-length CD44 variant structures will be used for the transfection of eukaryotic cells [e.g. non-metastatic human breast epithelial cell line (HBL 100)-provided by Dr. Mien-Chie Hung at M.D. Anderson Cancer Center, Texas and shown to display only CD44E but not CD44v or CD44s (preliminary observations)]. The transfected cells will be assayed for the expression of a variety of functions such as adhesion and metastasis as described below.

(a) Measurement of Cell Surface Expression of CD44 Variant (CD44v) Isoforms In Human Breast Epithelial Cell Line Transfected With Various CD44v (e.g. CD44v-I, CD44v-II and CD44v-III) cDNAs:

(1) Flow Cytometric Analysis: Human breast epithelial cell line (HBL 100) transfected with various CD44v (e.g. CD44v-I, CD44v-II and CD44v-III) cDNAs, either unfixed or fixed with 2% paraformaldehyde will be incubated with one of the following reagents [Fl-conjugated extracellular matrix materials (e.g. hyaluronic acid (HA), collagen type I, chondroitin sulfate and keratin sulfate) or Fl-anti-CD44v antibodies-according to the procedures described below] at 0° for 30 min. To establish that CD44v is the binding site for specific extracellular matrix materials, we also plan to incubate specific anti-CD44v antibodies prior to the addition of Fl-conjugated extracellular matrix materials. The fluorescence labeled cells will then be analyzed by a flow cytometer using single or multiparameter analysis.

(2) Radio-Ligand Binding Assays: Human breast epithelial cell line (HBL 100) transfected with various CD44v (e.g. CD44v-I, CD44v-II and CD44v-III) cDNAs (1×10^6 cells/ml) will be incubated with one of the following reagents ([^{3}H]-extracellular matrix materials, e.g. hyaluronic acid (HA), collagen type I, chondroitin sulfate and keratin sulfate or ^{125}I-anti-CD44v antibodies) at 22° for 60 min with gentle shaking. Following incubation, cells will be washed and the cell bound radioactivity will be determined. Non-specific binding will be determined in the presence of a 100-fold excess of unlabeled ligands. The involvement of certain CD44v in extracellular matrix material binding will be determined by incubating anti-CD44v antibodies prior to adding radioactively labeled ligands.

(b) Cell Adhesion Assays: Adhesion is mediated through specific tumor cells and endothelial cell surface components, which influence the organ preference of metastatic tumor formation. CD44v proteins are modified by N- and O-glycosylations and sialylation. In order to determine the role of various CD44v isoforms in adhesion, we plan to perform the adhesion assays described as follows: First, human breast epithelial cell line (HBL 100) transfected with various CD44v (e.g. CD44v-I,

CD44v-II and CD44v-III) cDNAs (1 x 10^6 cells/ml) (5 x 10) will be incubated in various extracellular matrix material [e.g. hyaluronic acid (HA), collagen type I, chondroitin sulfate and keratin sulfate]-coated dishes at 22° for 30 min. In some cases, cells will be pre-incubated with various anti-CD44v antibodies to block certain extracellular matrix material-mediated cell adhesion. The measurement of extracellular matrix material-mediated cell adhesion or aggregation will be examined using a light microscope as described previously. Other extracellular matrix materials such as proteoglycan/corelinked proteins will also be used for cell adhesion assays using cells expressed various CD44v mutant proteins. In addition, the binding for cells [e.g. human breast epithelial cell line transfected with various CD44v mutant cDNAs] to high endothelial venules in frozen sections of peripheral lymph nodes and Peyer's patches will also be carried out according to the procedures described previously. Briefly, human breast epithelial cell line (HBL 100) transfected with various CD44v (e.g. CD44v-I, CD44v-II and CD44v-III) cDNAs will be incubated in DME with 5% calf serum and 10mM HEPES with mild rotation at 37° for 30 min on fixed frozen sections of BALB/c peripheral lymph node and Peyer's patches (2.0-3.0 x 10^6 cells in 100µl/section). The slide will be fixed in cold PBS containing 2% glutaraldehyde. At least three sections containing multiple lymph nodes or Peyer's patches will be counted for each condition or sample. The results will be expressed as the number of bound human breast epithelial cells (expressing CD44v) per high endothelial venule. It should be noted that the binding specificity of human breast epithelial cells (expressing CD44v) is conserved across species barriers, thus allowing the use of mouse sections for assays with human cells. The results of these proposed cell adhesion experiments will indicate whether there are changes in adhesion properties during various CD44 variant isoform expression.

In addition, we will create additional deletion and/or site-directed mutants that lack particular sequences or adhesion-binding sites in the extracellular domain or in the cell transmembrane domain of CD44v structure to further dissect the involvement of N-/O-glycosylation, sialylation and the

hydrophobic membrane spanning domains in the expression of CD44v adhesion for certain extracellular matrix components such as hyaluronic acid, collagen, chondroitin sulfate, keratin sulfate and other proteoglycan core/linked proteins using the adhesion assays described above. These mutants will also be used in the metastatic assays (as described below) in order to correlate the up or down/regulation of adhesion with metastatic behavior.

(c) Experimental Metastatic Assay: Six-week-old pathogen-free female nude mice (Harlan Sprague-Dawley, Indianapolis, IN) will be quarantined for 1 week and then used for the assays. Seven to 10 mice per experimental group will be injected with 1 x 10^5 cells [e.g. human breast epithelial cell line (HBL 100) transfected with various CD44v - CD44v-I, CD44v-II and CD44v-III cDNAs] in 0.1ml of phosphate-buffered saline (PBS) via the lateral tail vein at day 0. Mice will be killed using carbon dioxide at 21 days after injection, and the number of lung metastases will be determined. Only those lung tumors >1mm in diameter will be counted according the procedures described previously. Animals will also be examined for extrapulmonary metastases. This assay will allow us to verify whether breast epithelial cells expressing various CD44v isoforms are capable of inducing metastasis as those described in rat tumor metastasis.

(B) To generate polyclonal and monoclonal antibodies against "fusion" proteins containing CD44 variant exons (related to metastatic breast carcinomas) in order to establish a useful metastatic marker for breast cancer:

The procedures for the cloning of "fusion" proteins containing CD44 variant exons (related to metastatic breast carcinomas) are as follows:

(a) Cloning of pRSET Fusion Proteins: The whole variant region of human breast carcinoma-related CD44 variants (e.g. CD44v-I, CD44v-II and CD44v-III) will be first cloned into pCRII vector using a one step PCR cloning (TA cloning) kit from Invitrogen Corporation. Its nucleotide sequence will be determined by the dideoxytermination method as described above. Subsequently, the variant

region will be cloned into pRSET(A,B,C) prokaryotic protein expression vectors developed by Invitrogen Corporation. This expression system offers high level transcription from the bacteriophage T7 promoter, and recombinant fusion proteins can be one-step purified with ProBond™ resin and subsequently cleaved with enterokinase. Since pRSET vectors contain multiple cloning sites in three different reading frames, this would allow variant regions of CD44v cDNA to be readily inserted in frame with the Xpress™ N-terminal peptide. This short leader peptide has high affinity for ProBond™ metal resin permitting one step purification of fusion proteins from crude bacterial cell lysates. Specifically, variant regions of CD44v cDNA with the addition of exon5 and 15, will be directionally cloned into pRSET prokaryotic expression vector as follows: the constructs (pCRII/CD44 variant region) from the previous section are first digested with HpaI endonuclease generating blunt ends at the 5' side of CD44 variant cDNA fragments. Then, these linearized plasmids are digested with EcoRI, resulting in the excision of the whole CD44 variant regions with blunt ends at 5' side and 5'overhang at 3' side. Similarly, appropriate pRSET (A,B, or C) vector will be first digested with BamHI followed by incubation with Klenow fragment DNA polymerase and dNTPs to create blunt ends. The linearized vector is digested with EcoRI restriction endonuclease. Finally, excised CD44 variant regions are ligated directionally to pRSET plasmid vectors with T4 DNA ligase in the presence of ATP. The resulting constructs code for Xpress™ N-terminal peptide fusion proteins of variant regions of CD44v molecules.

In order to obtain subclones of the variant regions that can be used for the generation of variant-specific antibodies, these variant exons are cloned into pRSET vectors using appropriate restriction sites or alternatively employing PCR technique where unique 5' (left) oligoprimer is designed with the addition of HpaI site and 3' (right) primer with EcoRI site. These constructs (pRSET/CD44 variant region) are used to produce recombinant fusion proteins from prokaryotic cells. The recombinant protein fused to Xpress™ N-terminal peptide will be purified as described

above and further utilized for immunization of rabbits or mice to generate respective region-specific antibodies of CD44v proteins.

(b) Generation of Polyclonal and Monoclonal Antibodies Specifically Against CD44v (e.g. CD44v-I, CD44-II and CD44-III): The procedures used for generating polyclonal and monoclonal antibodies against "fusion" proteins containing CD44 variant exons (e.g. CD44v-I, CD44v-II and CD44v-III) will be the same as these described previously. Standard antibody characterization methods such as ELISA, immunoblotting and immunonoprecipation will be used to verify the specificity and the titers of polyclonal and monoclonal antibodies against various CD44v as described previously. In addition, we plan to carry out a simultaneous localization of CD44s and CD44v, at both mRNA and protein levels, using state-of-the-art laser confocal microscope in order to determine whether there is a selective distribution (i.e. expression in a single cell type or a variety of cell types) of CD44v in breast cancer tissues during metastasis.

(1) <u>In situ</u> Hybridization Assays Using Immunofluorescence Labeling Techniques: CD44 variant exons unique to metastatic breast carcinomas will be cloned into a plasmid vector (e.g. pBluescript or pRCII vector). Then, antisense- and sense-specific CD44v and CD44s RNA probes will then be labeled with digoxigenin-UTP (RNA labeling kit, Boehringer Mannheim). <u>In situ</u> localization will be done at 42° overnight using breast cancer tissue sections or CD44v-transfectants prepared according to the procedures described previously. Sections will be washed and incubated with anti-digoxigenin antibody-conjugated to fluorescein (FITC) (20 μg/ml) for 1 h at room temperature. Slides will be washed briefly and examined using a laser scanning confocal microscope (MultiProbe 2001 Invert CLSM System, Molecular Dynamics).

(2) Simultaneous Localization of CD44v Proteins (e.g. CD44v-I, CD44-II and CD44v-III) And CD44s Using Double Immunofluorescence Staining Techniques: Breast cancer tissue sections or CD44v transfectants will be first fixed with 2% paraformaldehyde in PBS (0.1M

phosphate buffer and 0.15 M NaCl) for 1h at 4°. For simultaneous localization of CD44v and CD44s, sections will be incubated FITC-conjugated rabbit anti-CD44v antibody and Rh (rhodamine)-conjugated rat anti-CD44s at 4° for 1h. Samples will be washed briefly and examined by a laser scanning confocal microscope (MultiProbe 2001 Invert CLSM System, Molecular Dynamics). The results of both in situ hybridization and double immunofluorescence techniques will allow us to pinpoint whether various CD44v isoforms are derived from a single cell or multiple cell types in metastatic breast tumors. Furthermore, we plan to test the expression of various CD44 variant isoforms in a larger number of metastatic breast cancer samples using immunofluorescence staining as described above in order to establish a possible relationship between CD44 variant isoforms and the progression of human breast cancers.

Anticipated Results:

(i) The use of a variety of molecular biological techniques, including RT-PCR, DNA sequence analyses and in situ hybridization will provide rapid detection of the specific CD44 variant (CD44v) isoforms associated with metastatic breast carcinoma tissues.

(ii) The availability of various CD44v transfectants (using non-metastatic human and mouse mammary tumor cell lines) will provide us an opportunity to analyze CD44v-cytoskeleton interactions, adhesion functions and the associated metastatic behavior. Since the regulatory factors involved in controlling metastasis in breast cancer are not well established, the results of our proposed studies will offer new insights towards a better understanding of this important pathological phenomenon. This information will be very important for designing future treatments or for prevention of breast cancer metastasis.

(iii) A newly established laser scanning confocal microscopy facility and its variations for fluorescence and other applications will provide a powerful new technology of significant benefit to our proposed breast cancer research projects. A computer-assisted image analysis system will allow

a sophisticated immunocytochemical analyses to be obtained. In addition, this state-of-the-art technology will allow us to obtain better resolution regarding immunocytochemical localization of various CD44v and CD44s in thick specimens of breast tissues and better assessment of detailed spatial relationships between various CD44v isoforms and CD44s.

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APPENDIX:

Fig. 1. A representative RT/PCR and Southern Blot analyses of CD44 isoforms using RNAs isolated from human breast tissues. (A-D: normal breast tissues; E-K metastatic breast tumor tissues).

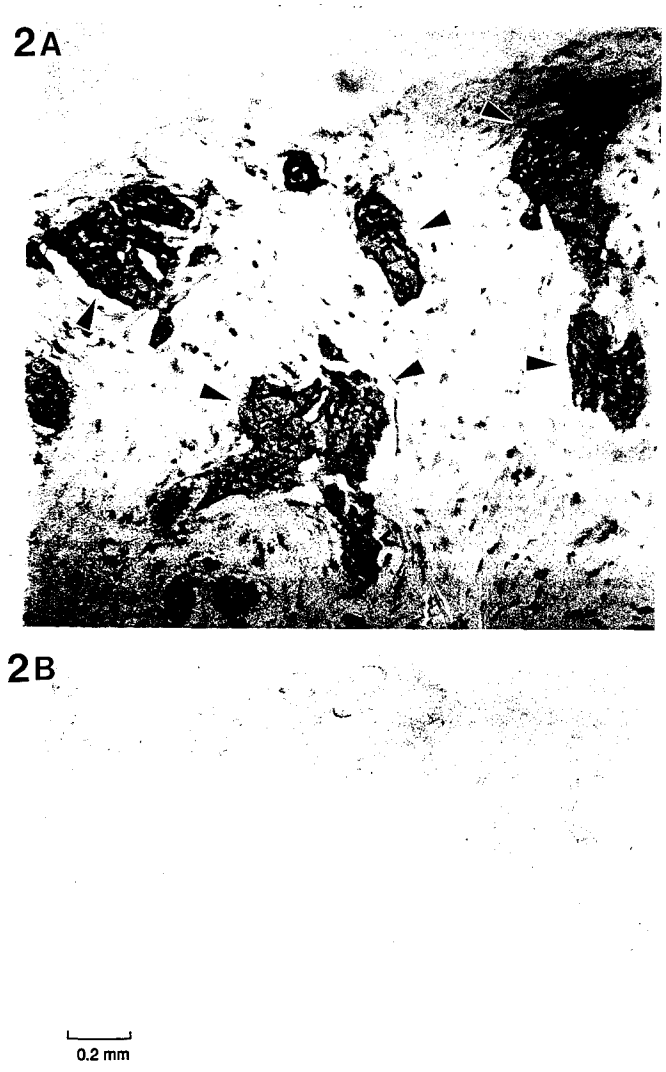

Fig. 2. Immunohistochemical staining of CD44 in metastatic breast carcinomas (A) and normal breast tissues (B).

A: Immunoperoxidase staining of CD44 in metastatic breast carcinoma tissues. [showing a preferential localization of CD44 at the plasma membrane region of the tumor cells (indicated by arrow heads)].

B: Immunoperoxidase staining of CD44 in normal breast tissues. [showing no detectable CD44 staining in these samples].

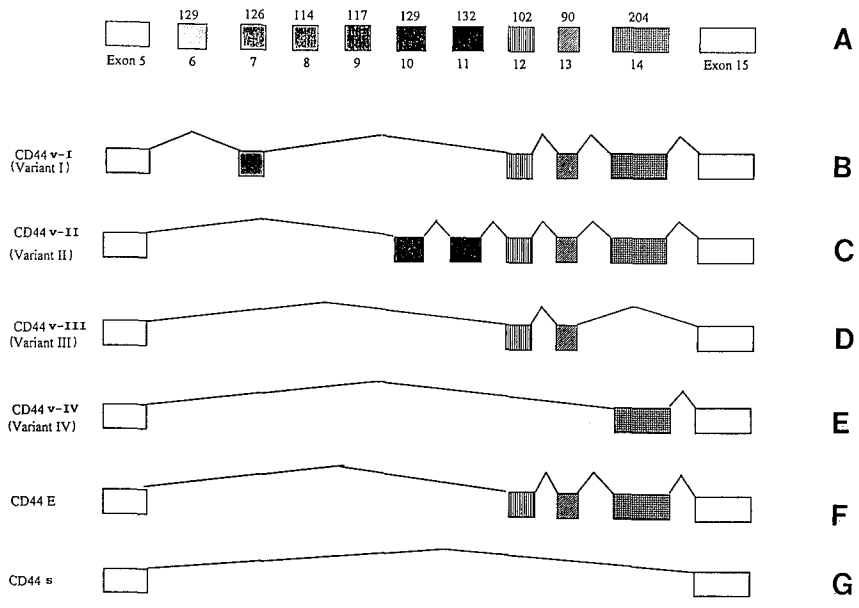

Fig. 3. Schematic exon map of CD44.
A: showing all 9 possible exon insertions (e.g. exons 6-14) between exon 5 and exon 15 of CD44.
B: showing exons 7,12,13 and 14 are inserted between exon 5 and exon 15 (designated as CD44v-I).
C: showing exons 10,11,12,13 and 14 are inserted between exon 5 and exon 15 (designated as CD44v-II).
D: showing exon 12 and 13 are inserted, but exon 14 is spliced out between exon 5 and exon 15 (designated as CD44v-III).
E: showing exon 14 is inserted between exon 5 and exon 15 (designated as CD44v-IV).
F: showing exons (e.g. insertion of exons 12, 13 and 14 between exons 5 and 15) encoding the epithelial form of CD44 (CD44E).
G: showing exons (e.g. no insertion of exons between exon 5 and 15) encoding the standard form of CD44 (CD44s).